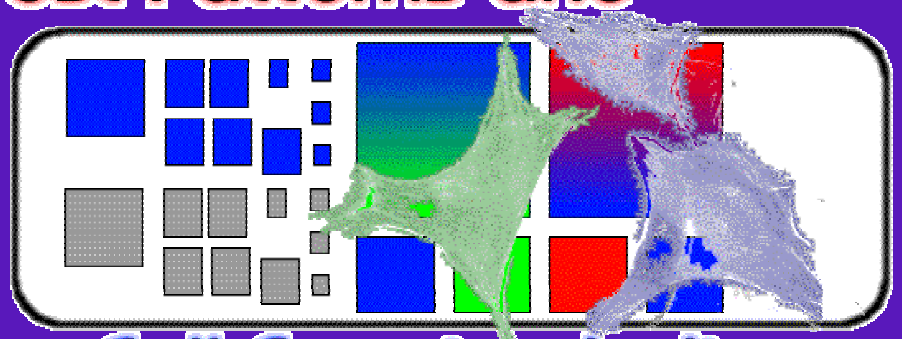
Metrology for Tissue Engineering

Test Patterns and



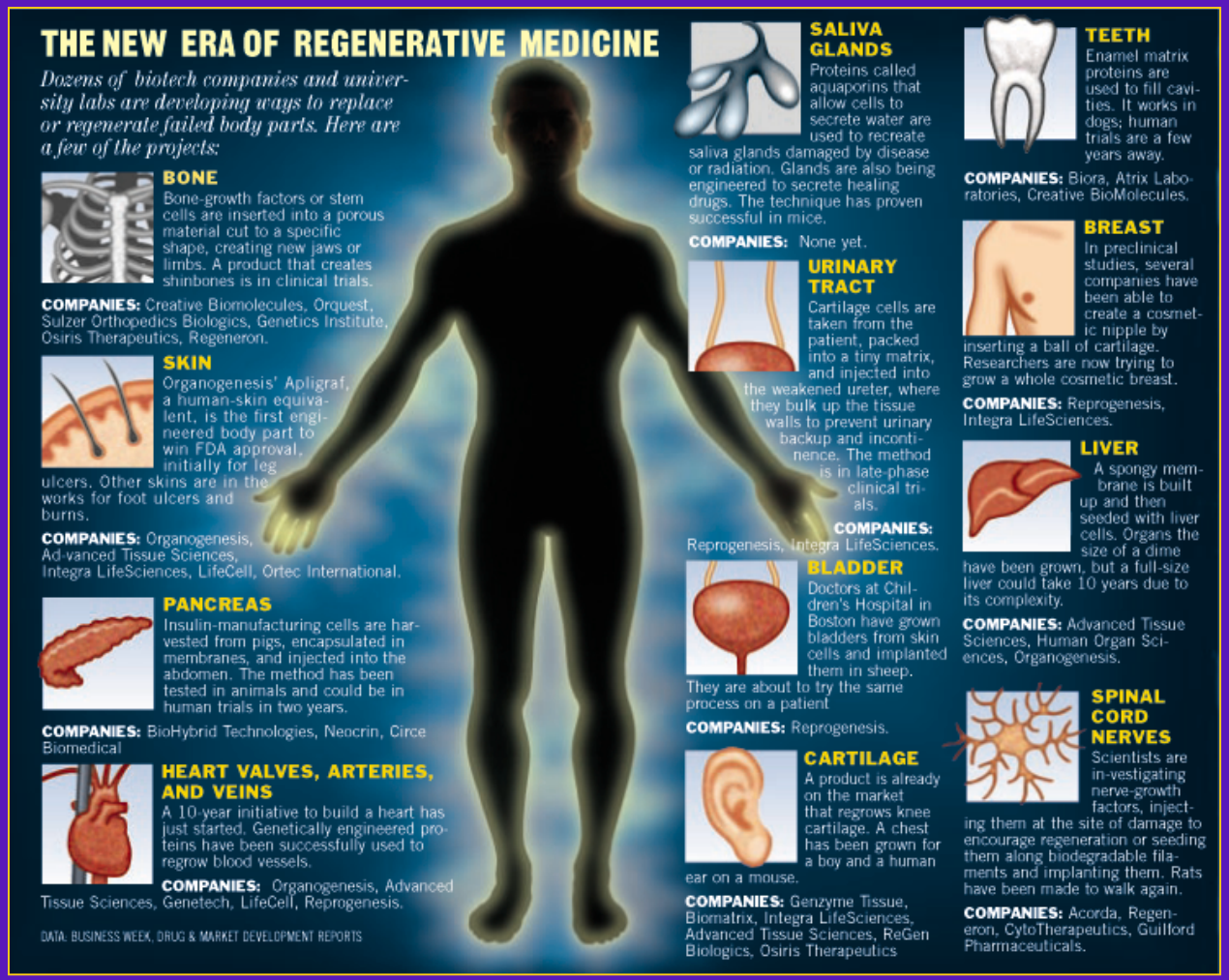
Cell Function Indicators

Scott Kennedy, Amit Sehgal, Newell Washburn (MSEL)
John Elliott, Kurt Langenbach, Anne Plant (CSTL)
Kenneth Yamada (NIH)
Peter Jones (Colorado)

NIST
National Institute of Standards and Technology
Technology Administration, U.S. Department of Commerce

Test Patterns and Cell Function Indicators

Tissue Engineering: Defining Regeneration

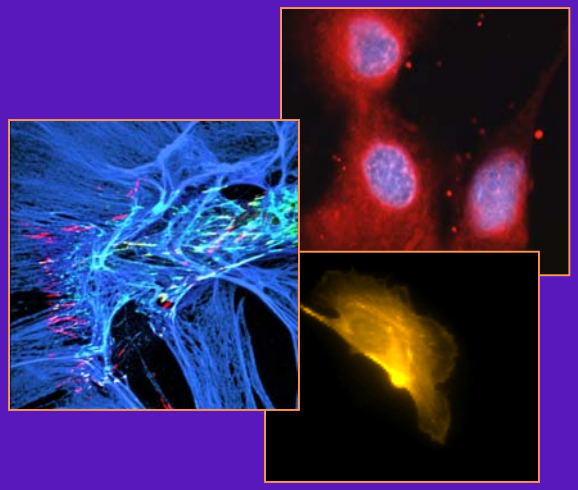


Generating functional replacements for missing or damaged tissue

Test Patterns and Cell Function Indicators

Engineering Tissue by Controlling Cellular Response

Cellular responses characterized by:
Proliferation
Differentiation
Matrix Production
Migration
Programmed Cell Death



Tissue engineering tools:
Scaffolds
Extracellular Matrix Proteins
Growth Factors
Gene Delivery

Achieving this goal requires understanding complex signals that drive cellular differentiation and organization

Test Patterns and Cell Function Indicators

Tissue Engineering: Current Products

- Skin substitutes
  - Apligraf (Organogenesis/Novartis)
  - Dermagraft (Smith-Nephew)
  - Dermal Regeneration Template (Integra Life Sciences)
- Cartilage engineering
  - Carticel (Genzyme)
  - Synvisc (Genzyme)
- Bone engineering
  - Smartscrew (Bionx Implants)
  - CMB401 (Integra Life Science/American Home Products)
- Dental tissue engineering
  - Biomend (Integra Life Sciences)

Integra Artificial Skin Dermal Regeneration Template
Collatape™ Collaplug™ Collacote™

ETHICON, INC. a Johnson & Johnson company

genzyme

OSIRIS

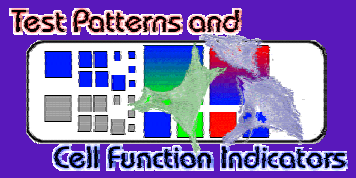
CYTOMATRIX CELL GROWTH SYSTEM

INTEGRA LIFESCIENCES HOLDINGS CORPORATION

Over 70 start-up companies total*

*[Lysaght MJ, Reyes J. Tiss Eng 7, 485-493 (2001)]

Industry needs a measurement infrastructure

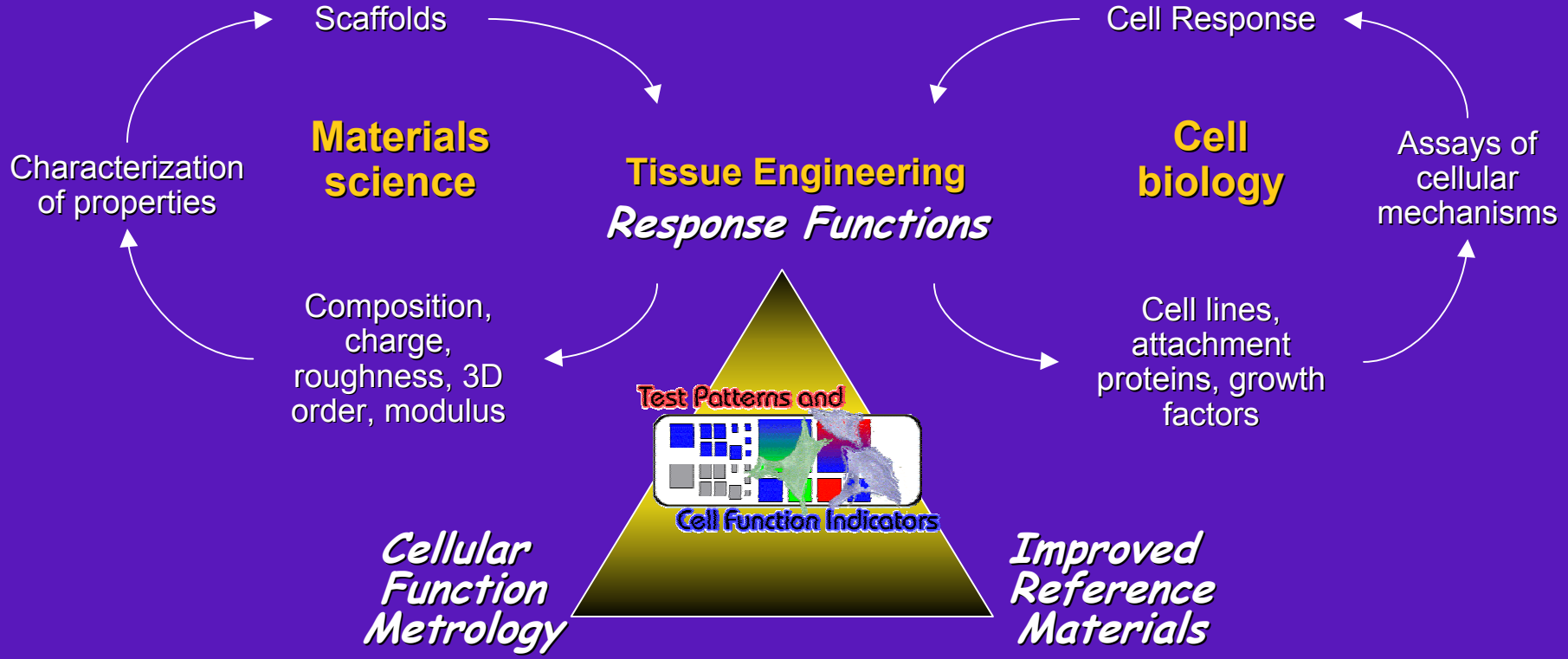


The FDA Response: Office of Cellular, Tissue, and Gene Therapies

From the OCTGT Functional Statement:

[The Office of Cellular, Tissue, and Gene Therapies] plans and conducts research related to the development, manufacture, testing, and activities of cellular, tissue, and gene therapy products, including those related to AIDS and those prepared by genetic engineering and synthetic procedures, in order to develop and maintain a scientific base for establishing standards designed to ensure the continued safety, purity, potency and effectiveness of cellular, tissue, and gene therapy products.

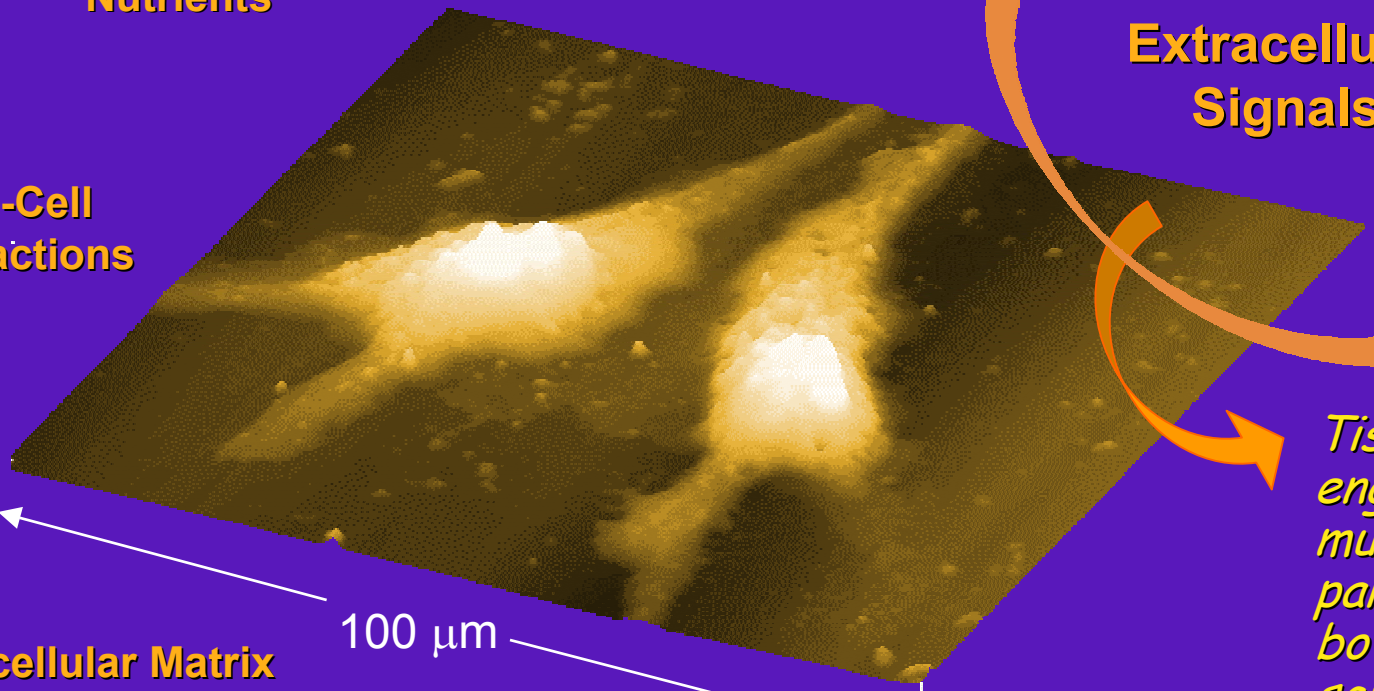
Metrology for Tissue Engineering



Couple materials science and cell biology expertise to prepare reference materials of varying surface properties, cells that signal their functional state, and maps of cell responses

Test Patterns and Cell Function Indicators

Measurements at the Bio/Material Interface

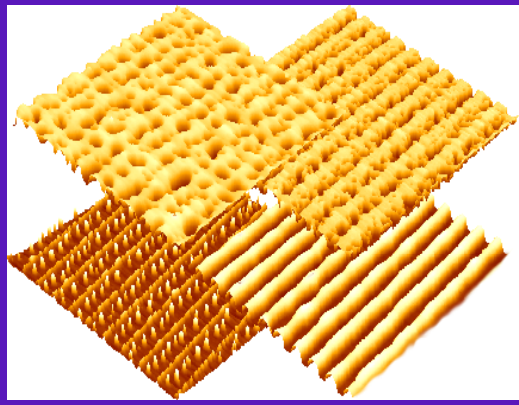


Tissue engineering must address parameters from both materials science and cell biology

Correlating Materials and Biology: The Deliverables

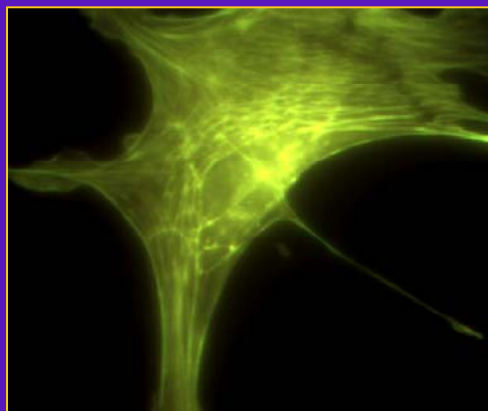
Materials

- Polymeric reference materials to replicate real processing variables and direct cell response
- Reference biomimetic materials to validate indicator cell response



Indicator Cells

- Develop genetically engineered cells for use as reporters of biocompatibility
- Demonstrate use of indicator cells of different tissue types for rapid screening of biomaterials



Test Patterns and Cell Function Indicators

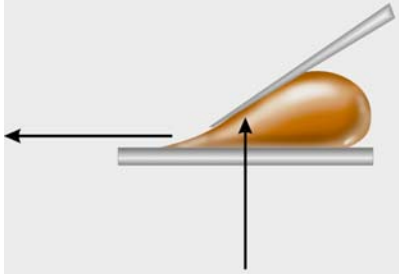
- Correlate cell response to materials properties with improved measurement methodologies

Test Patterns and Cell Function Indicators

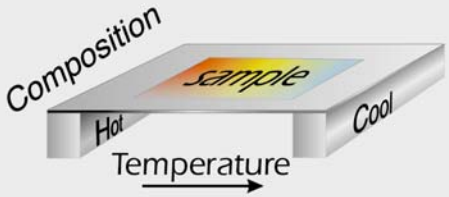
Gradient Libraries: Spanning Material Parameter Spaces

Library Preparation

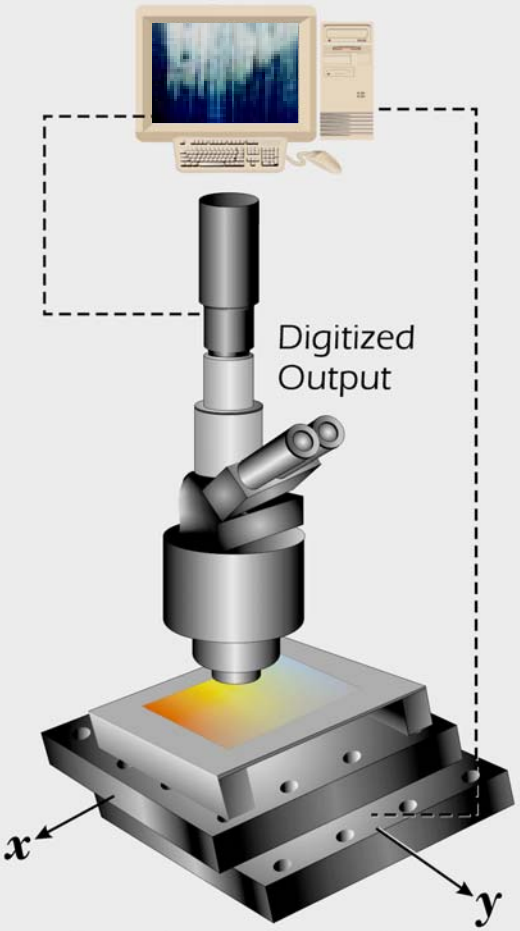
Composition Gradient via Flow Coating



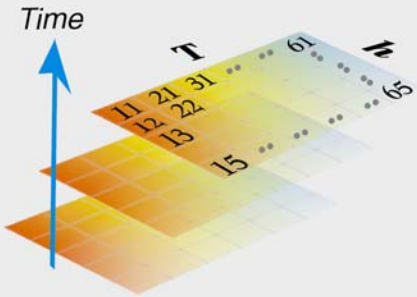
Polymer Film on Temperature Gradient Stage



Automated Data Acquisition



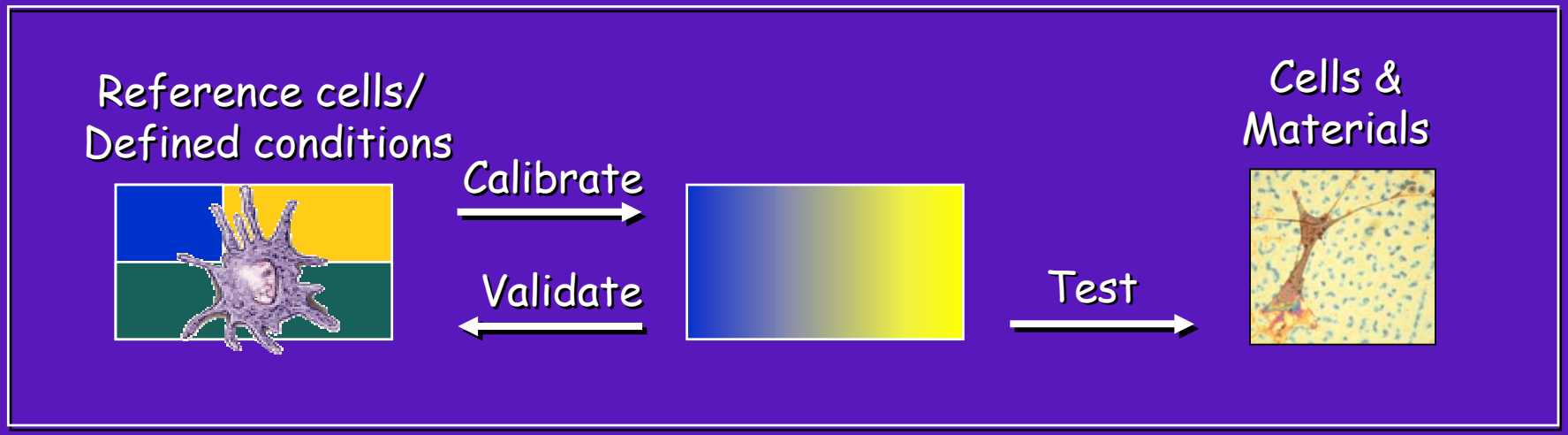
Informatics



- Divide Sample into Virtual Array
- Optimize Cell Size
- Process and Analyze Images
- Map Phase Behavior

Test Patterns and Cell Function Indicators

Metrology Chain for Gradient Libraries



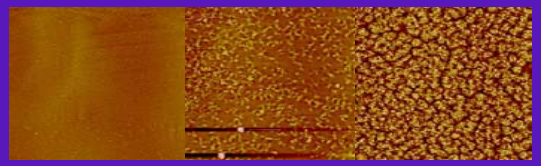
Using gradient libraries that model manufactured materials or control cellular response:

- Validate processing techniques.
- Test uncharacterized cells and measure response.
- Calibrate indicator-cell response.

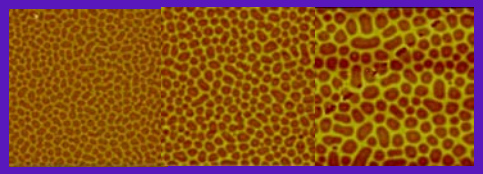
Gradient library methodology with defined cell culture conditions can be used to validate cells and materials as a part of a complete metrology chain for tissue engineering.

Test Patterns and Cell Function Indicators

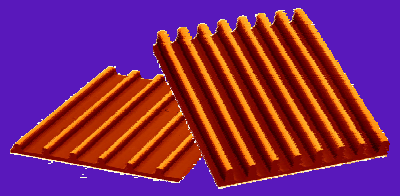
Biomaterial Gradient Libraries



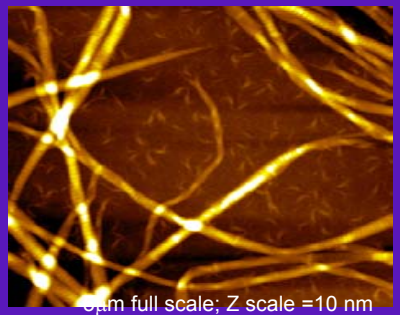
Polymer Crystallinity
(nm roughness)



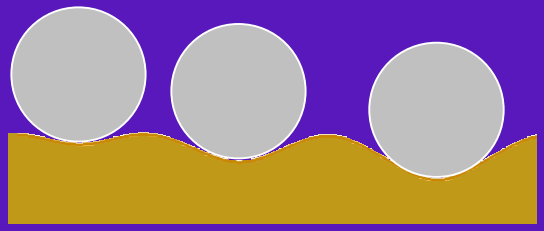
Polymer Blend Morphology
(μm roughness)



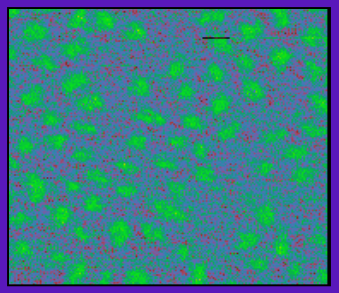
Surface patterns
(μm features)



Collagen Organization
(ECM model)



Hydrogel Modulus
(cellular mechanics)

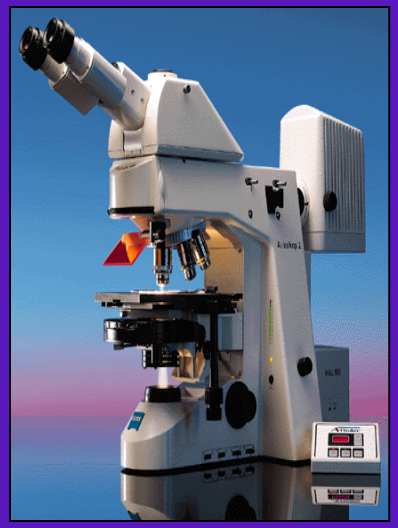


Signaling-Peptide Density
(nm organization)

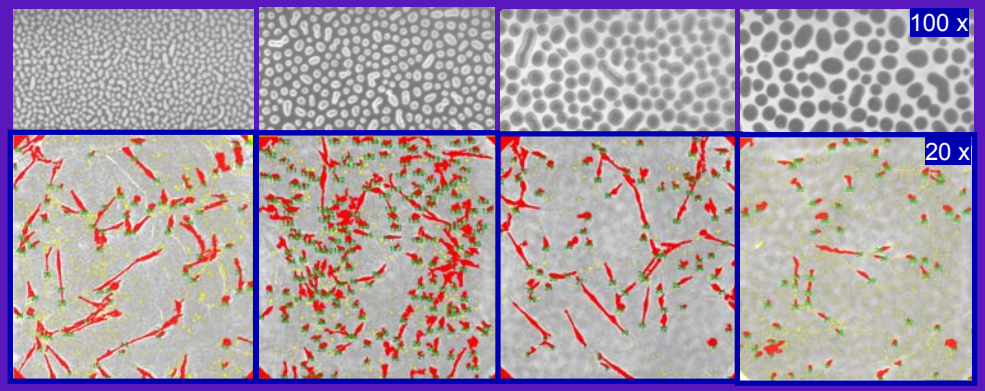
The performance of tissue engineered medical products is determined by both materials and processing conditions. Gradient libraries are capable of modeling both these parameters.

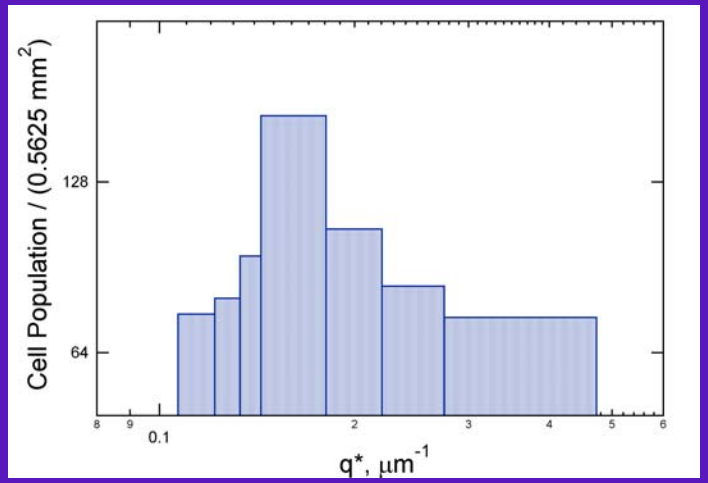
Test Patterns and Cell Function Indicators

Automated Microscopy



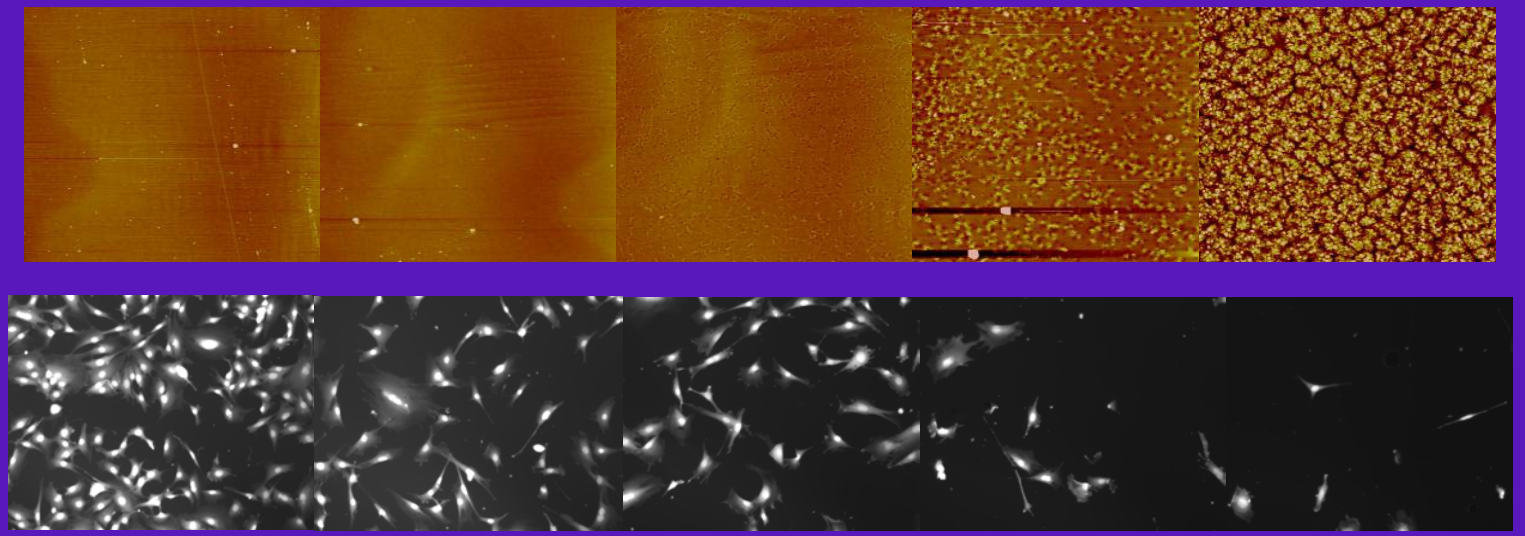
- Spatially-addressable microscopy to probe two-dimensional array
- Fluorescent staining used to selectively image different cellular components
- Image analysis software for quantification of cellular response





Test Patterns and Cell Function Indicators

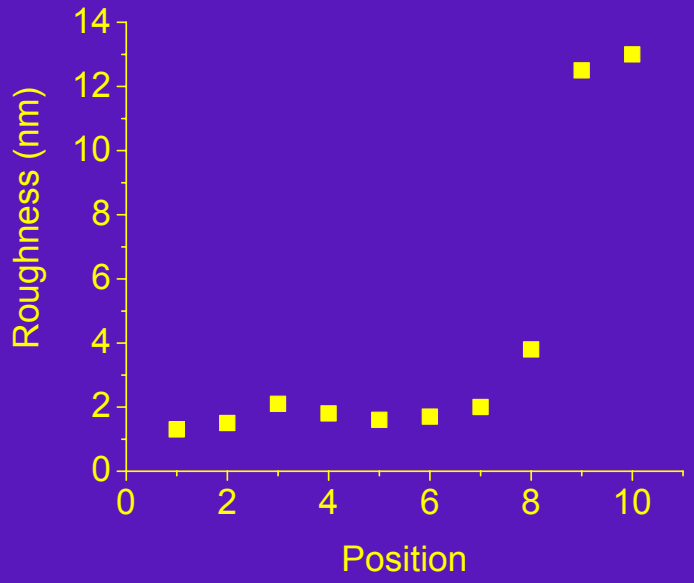
Cell Proliferation on Crystallinity Gradients

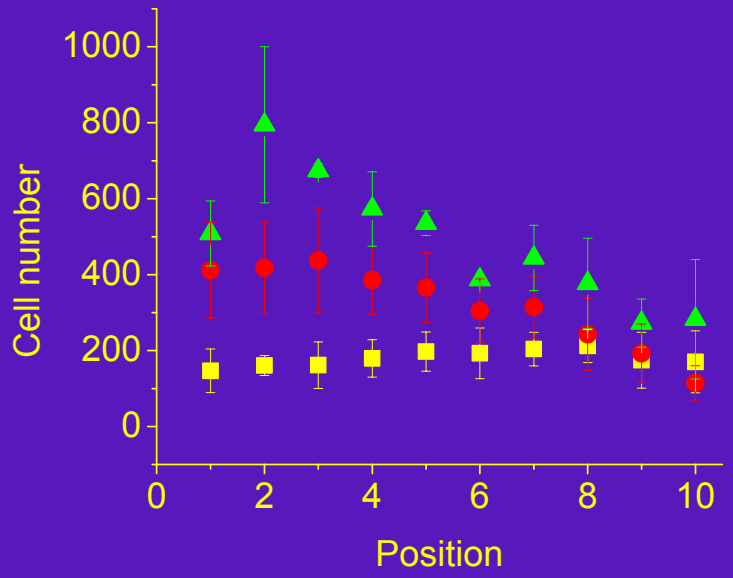


- Crystallinity gradient poly(L-lactic acid) film produced using temperature gradient.
- Osteoblasts cultured for 1, 3, and 5 d.
- Proliferation measured with automatic fluorescence microscopy.

Test Patterns and Cell Function Indicators

Quantitative Proliferation Data from Crystallinity Gradient Library





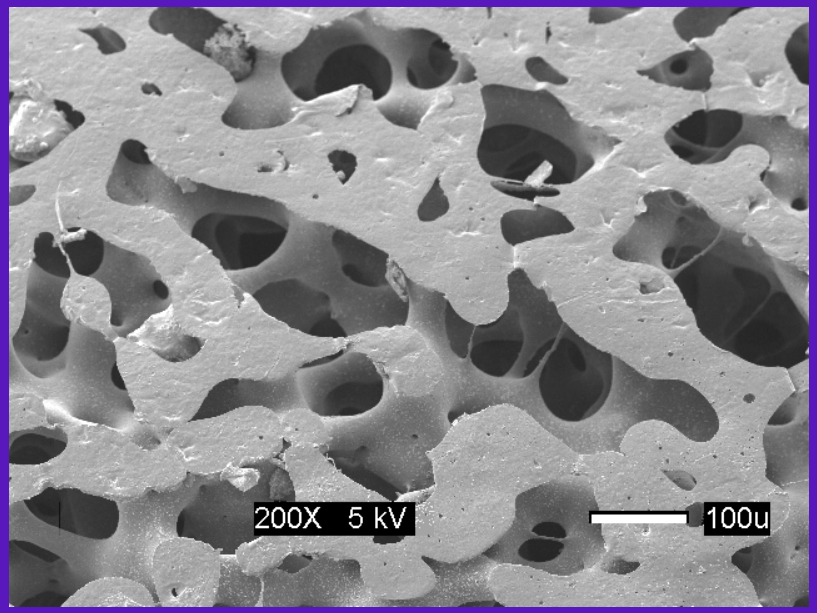
Gradient libraries provide the dependence of cellular response as a function of material parameters.

Test Patterns and Cell Function Indicators

Tissue Engineering Scaffolds

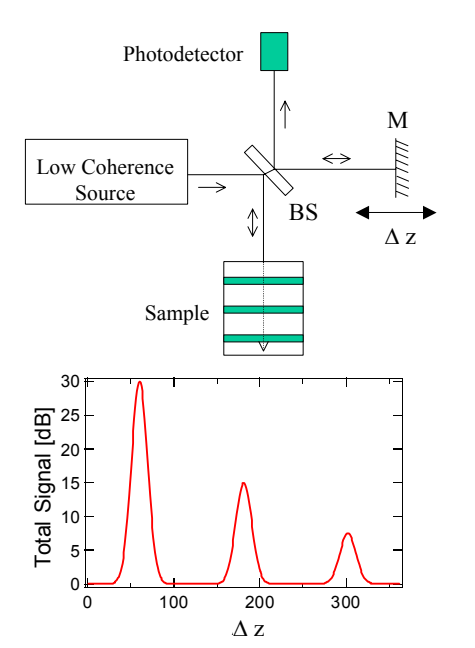
Scaffold Parameter Space

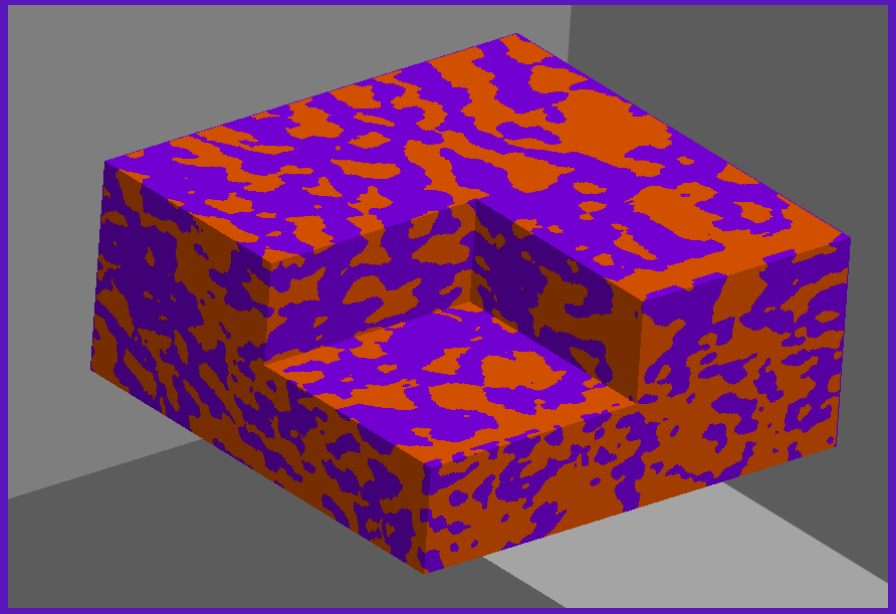
- Polymer mechanical properties
- Polymer surface properties
- Polymer degradation kinetics
- Local geometry
- Characteristic length scales
- Anisotropy



Methodologies are needed for measuring cellular response to scaffold structure and material properties.

Optical Coherence Tomography



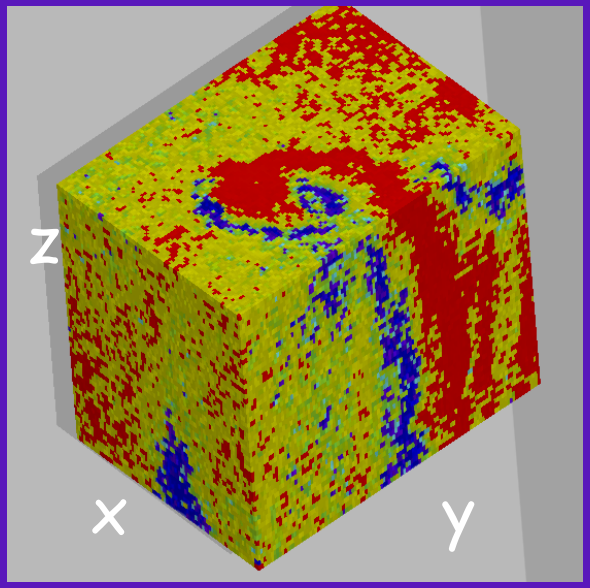


OCT is capable of providing three-dimensional structural information at micrometer-resolution, even in highly scattering media.

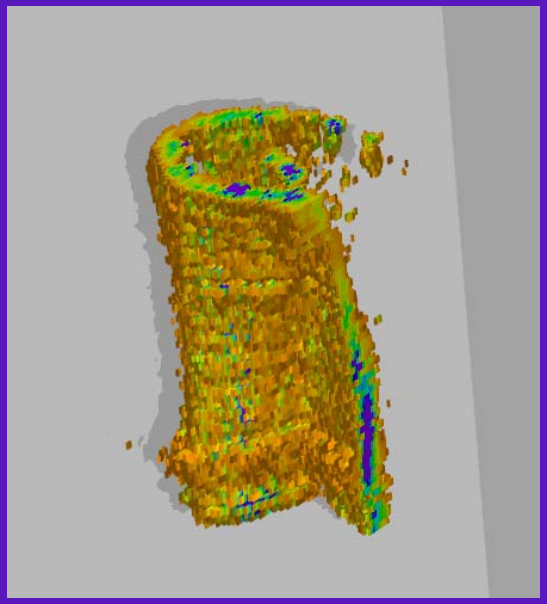
Test Patterns and Cell Function Indicators

Functional Imaging: Coupling Fluorescence and OCT

z=220 μm
x=160 μm
y=220 μm



OCT



Fluorescence image of stained cells in scaffold

Simultaneous maps of scaffold structure and tissue development.

Correlating Materials and Biology: The Deliverables

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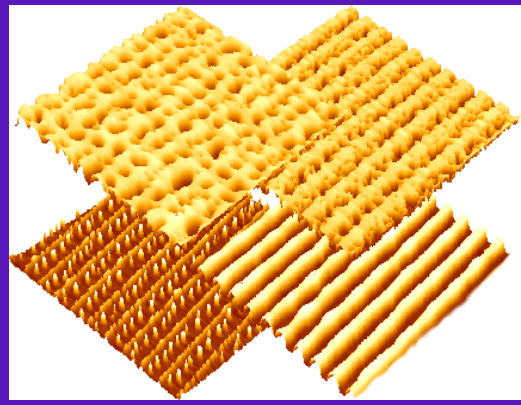
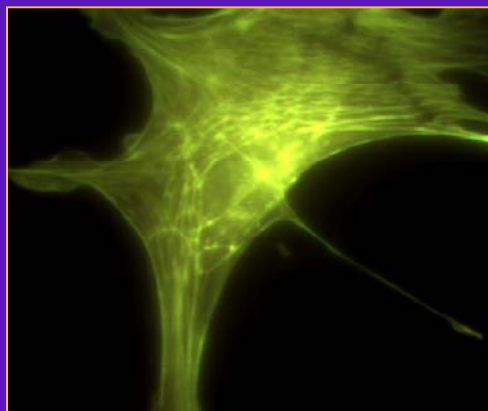
- Reference biomimetic materials to validate indicator cell response



Indicator Cells

- Develop genetically engineered cells for use as reporters of biocompatibility
- Demonstrate use of indicator cells of different tissue types for rapid screening of biomaterials



Test Patterns and Cell Function Indicators

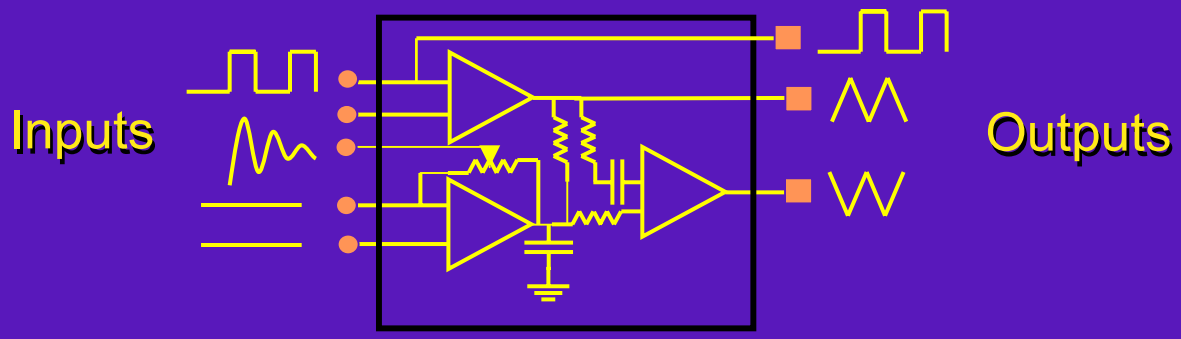
- Correlate cell response to materials properties with improved measurement methodologies

Test Patterns and Cell Function Indicators

Systems Analysis Approach to Cell Biology

Electronics

The Black Box



Systems Biology

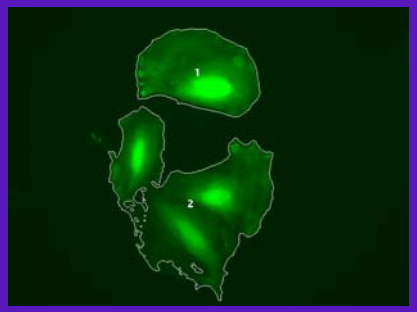
The Indicator Cell

Cell-based indicators signal the result of a response to microenvironment.

Test Patterns and Cell Function Indicators

Indicator Cells for Tissue Engineering

- Indicator Cells will provide a metric for rapidly assessing:
  - Cell response to new materials
  - Effect of materials processing/handling
  - Shelf life of materials
  - In vitro/In vivo biological response: inflammation, cell growth, biocompatibility, etc.
  - Cell response in 3-D matrices



We will assist the development of TE products by providing indicator cells that allow real time evaluation of whether cells will proliferate, differentiate or apoptose when exposed to a new biomaterial.

Test Patterns and Cell Function Indicators

Metrology Chain for Indicator Cells

The Metrology Chain

Using biomarkers that report cell response:

- Verify indicator-cell response.
- Calibrate response on reproducible defined materials.
- Test unknown materials and validate response on known materials.

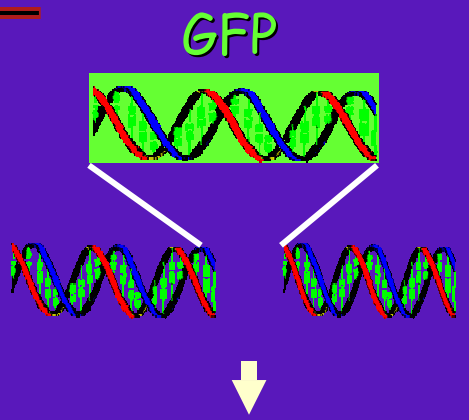
We will establish a metrology chain: NIST-validated markers used with NIST-validated conditions allows reliable assessment of new biomaterials.

Test Patterns and Cell Function Indicators

Designing Indicator Cells

Identify genes, cell type
Procure DNA
Engineer artificial gene

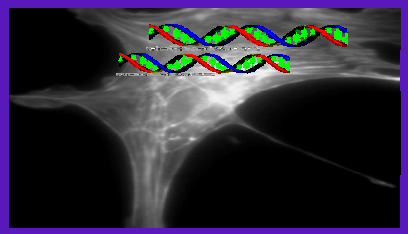
- Verify DNA integrity: DNA sequencing, restriction enzyme analysis



Consultations on biomarkers
- Mark Pittenger, Osiris
- Glen Prestwich, Echelon
- J. Mansbridge, Advanced Tissue Sciences
- Peter Davies, IME UPenn
- Peter Jones, UCHSC
- R. Valentini, Cell Based Delivery

Transfect cells
Select clonal populations

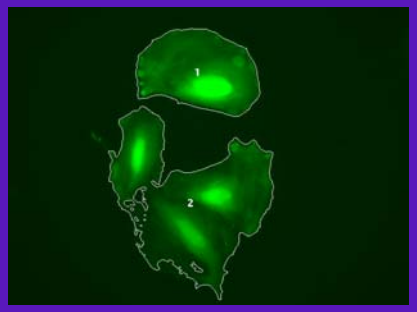
- Verify DNA uptake:select for specific antibiotic resistance



Transfection vectors
- BD Biosciences / Clontech

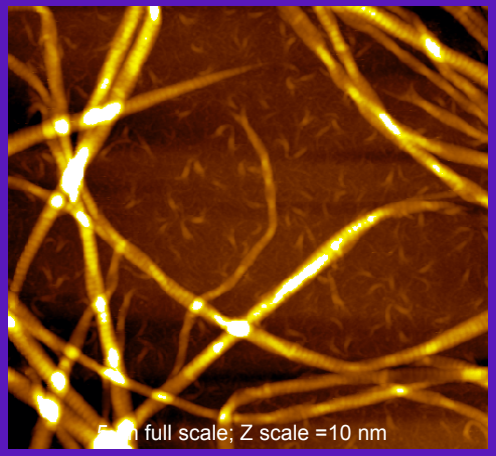
Establish conditions for gene activation

- Confirm gene activation: Northern and Western blot analysis, immunohistochemistry

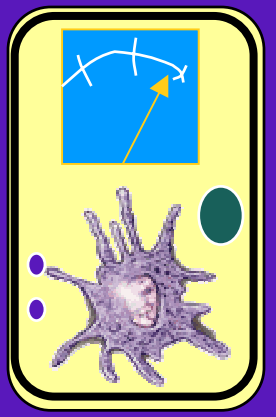


Verify signaling pathway
- Peter Jones, UCHSC

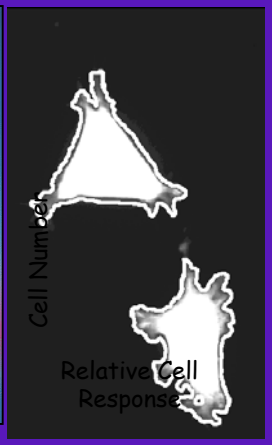
Calibrating Indicator Cells



Reference surfaces of extracellular matrix (ECM) proteins



Calibrate cell response on known reproducible surface

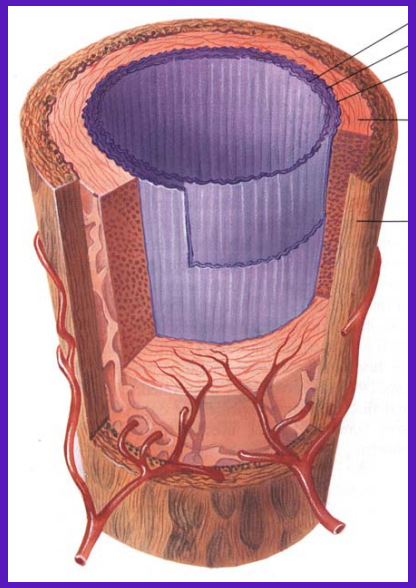


Quantitative fluorescence microscopy

Quantitative measurement of cell indicator response to reference conditions allows validation of indicator cells in other laboratories.

Test Patterns and Cell Function Indicators

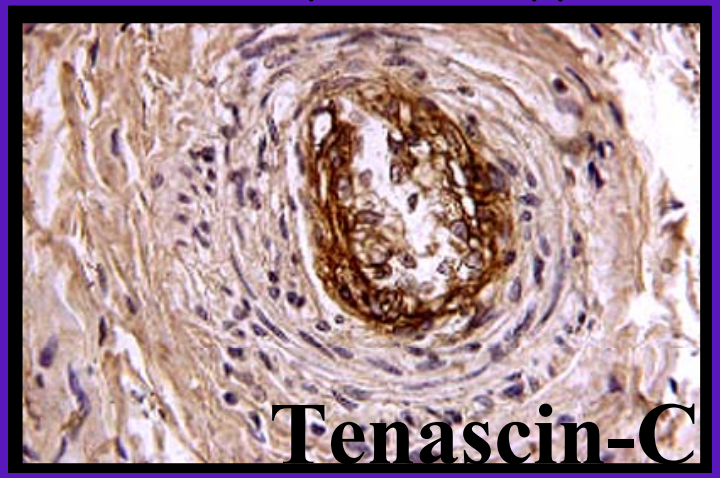
Indicator Cells in Practice: Smooth muscle cell proliferation in response to injury



Muscularized Artery

Injury

Blocked Artery from Hypertension



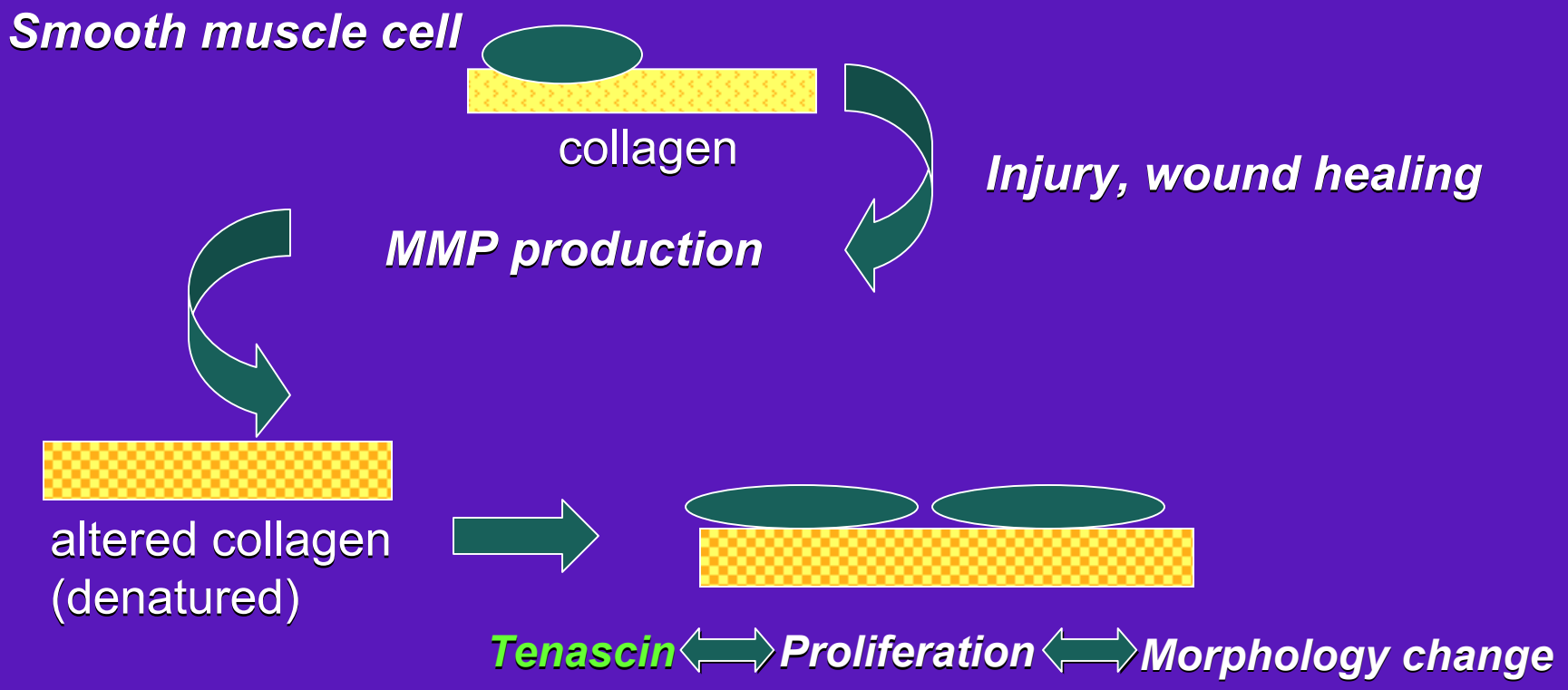
Courtesy of Peter Jones, UCHSC

Need for SMC Proliferation Control in TE

- Arterial Implants
- Synthetic Blood Vessels

Test Patterns and Cell Function Indicators

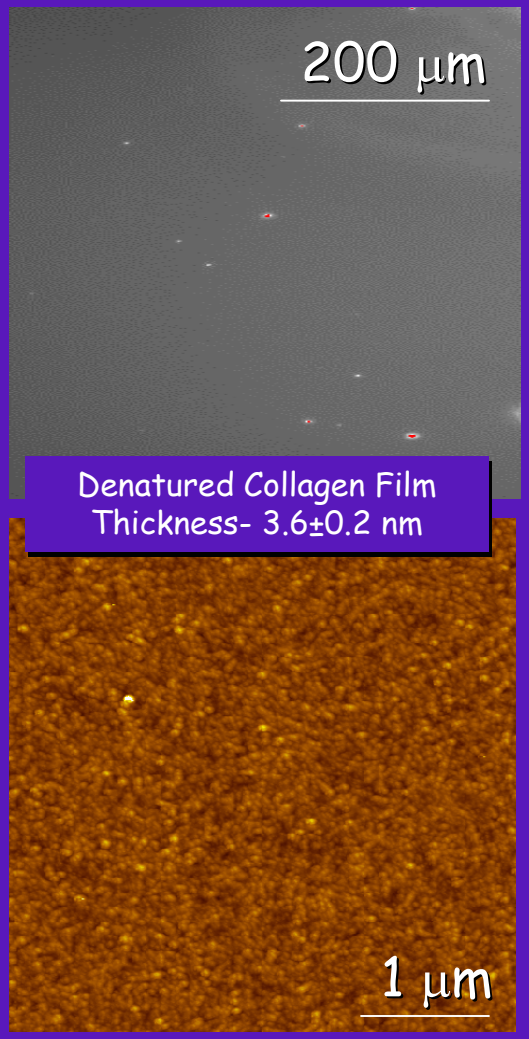
Smooth muscle cell proliferation in response to injury: Model System



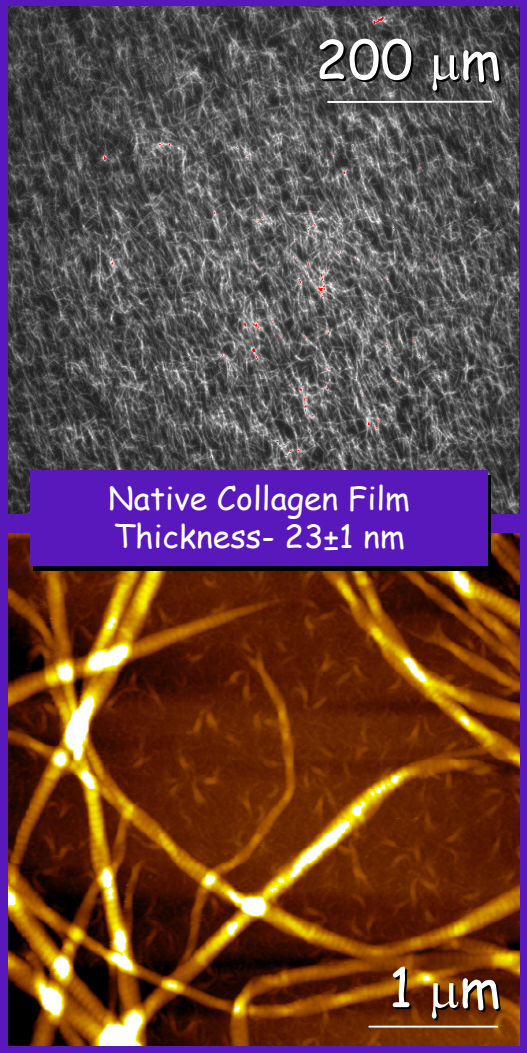
Several criteria are evaluated and used to validate the appropriateness and reliability of an indicator gene.

Test Patterns and Cell Function Indicators

Reference Biomimetic Collagen Surfaces based on Self-Assembly



Denatured Collagen Film
Thickness- 3.6±0.2 nm



Native Collagen Film
Thickness- 23±1 nm

WHY THIN FILMS based on self-assembly ?

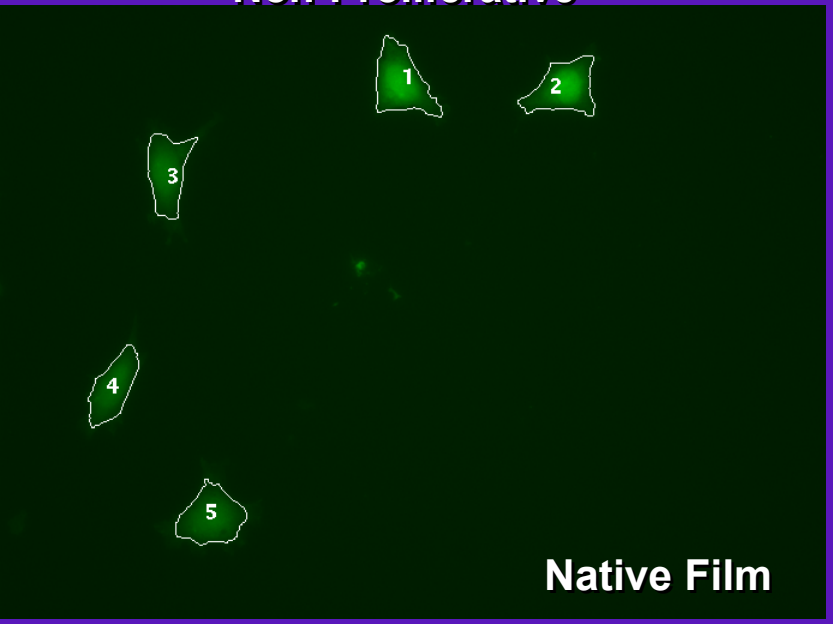
- **Currently no standardization of ECM surfaces.**
- **Removes surface inhomogeneity as a variable.**
- **Quantitative and reproducible.**
- **Could be fabricated in other laboratories.**

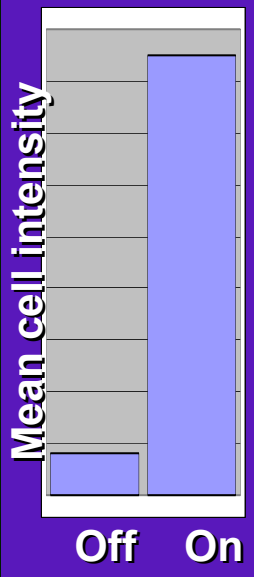
Reference materials will permit better inter-laboratory comparisons of data.

Validating Indicator Cell Response: GFP-Tenascin Expression

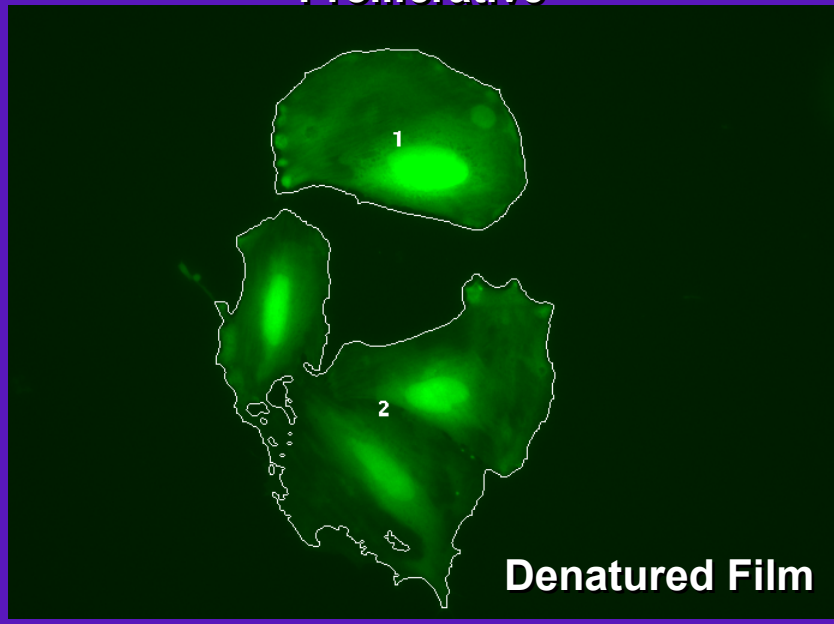
Assume that cell fluorescence ∝ GFP expression ∝ TN expression

Indicator Cell Status: Off
Non-Proliferative





Indicator Cell Status: ON
Proliferative

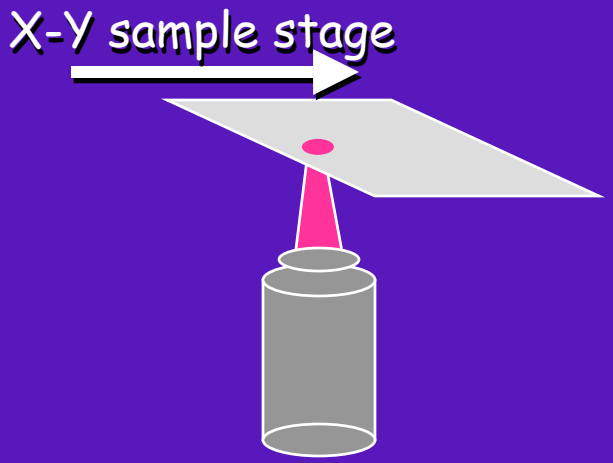


Optimization: Isolation of brighter single cell clones
Degenerate GFP
Transient transfection

Test Patterns and Cell Function Indicators

Validating Indicator Cell Response: Quantitative Microscopy

Fluorescence Microscope



Command

Image Analysis

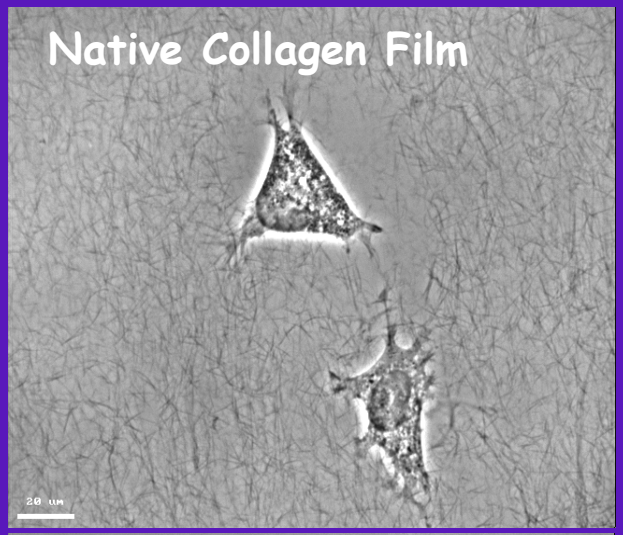
Data Output

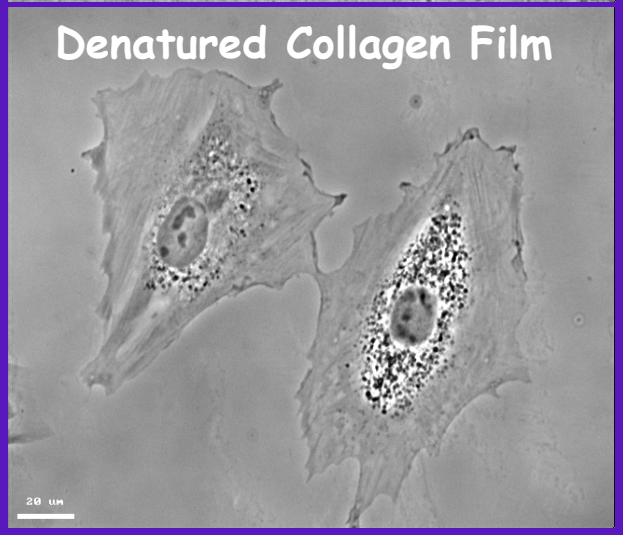
```
Cell Area:  3200
```

Automated quantitative microscopy allows unbiased collection of data from a statistically relevant number of cells.

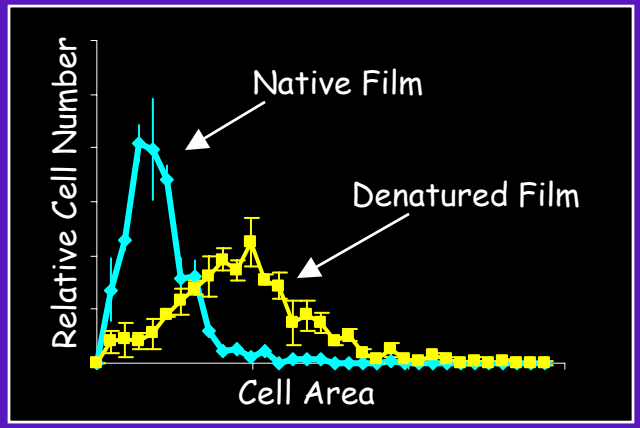
Test Patterns and Cell Function Indicators

Validating Indicator Cell Response: Morphology and Proliferation

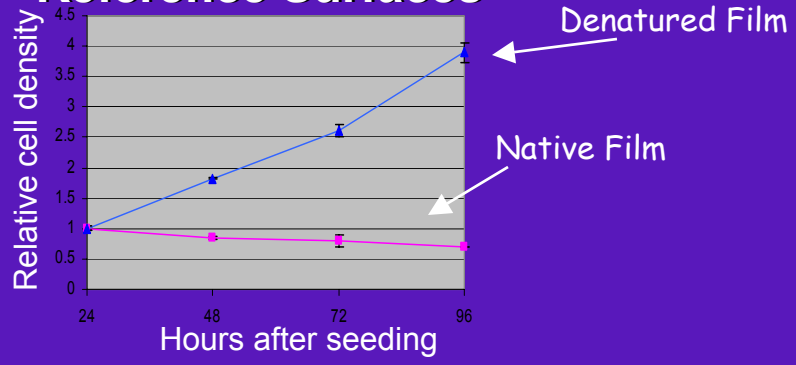




The mean size of SMCs, *and the distribution of sizes*, is highly reproducible on reference surfaces.



Proliferation rate is controlled by Reference Surfaces

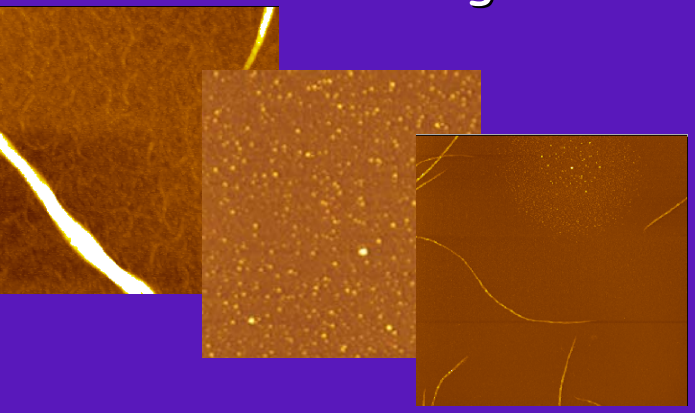


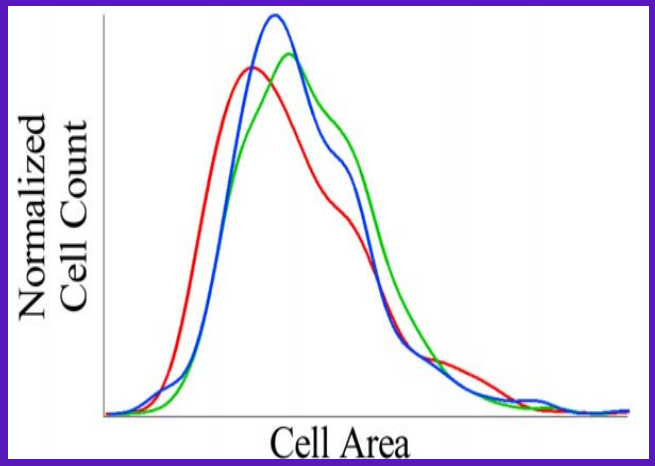
Morphology and proliferation measurements validate indicator cell response

Test Patterns and Cell Function Indicators

Statistics and Population Distributions

Cell Area on Collagen Films





Are these Distributions Similar?

	K-S test	χ^2 test	T-test	F-test
Red & Blue	>95%	No	No	>95%
Red & Green	>95%	No	No	No
Blue & Green	>95%	No	>95%	No

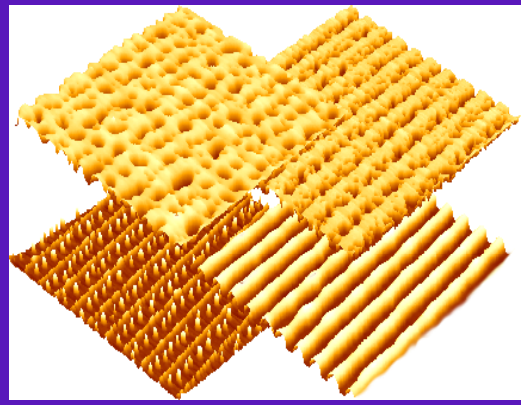
K-S = Kolmogorov-Smirnov

Identifying differences between population distributions may require rigorous statistical analysis

Correlating Materials and Biology: The Deliverables

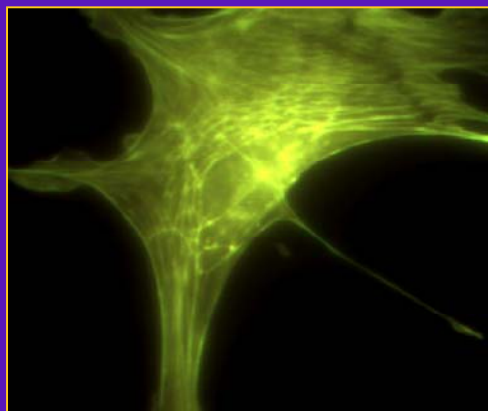
Materials

- Polymeric reference materials to replicate real processing variables and direct cell response
- Reference extracellular matrix materials to validate indicator cell response



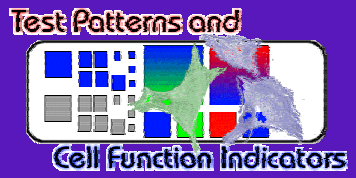
Indicator Cells

- Develop genetically engineered cells for use as reporters of biocompatibility
- Demonstrate use of indicator cells of different tissue types for rapid screening of biomaterials



Test Patterns and Cell Function Indicators

- Correlate cell response to materials properties with improved measurement methodologies



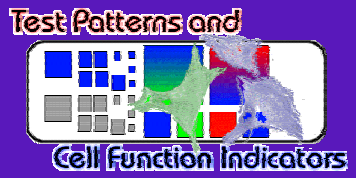
Accomplishments and Next Steps

Accomplishments

- Development of gradient libraries that impact cell proliferation.
- Construction and preliminary validation of GFP-Tenascin indicator
- Molecular biology expertise and facilities
- Peptide synthesizer set up to synthesize signaling molecules
- Microscopy methods for quantitative analysis of cellular response

Next steps

- Examine indicator cell response to polymer gradient materials
- Optimize TN promoter-GFP indicator cells
- Develop new promoter systems (for inflammation,oxidative damage,matrix production)
- Incorporate peptides into polymers and hydrogels to control cellular response
- Live cell quantitative microscopy



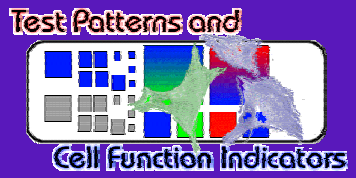
Accomplishments and Next Steps

Accomplishments

- Various cell lines established (bone, skeletal & smooth muscle, skin, kidney)
- Statistical methods developed for analyzing cell populations
- Established three-dimensional imaging methodologies.
- Construction of cell biology laboratories
- Reference surfaces of collagen
- Nine manuscripts in press, in preparation or submitted; 15 presentations made, 10 invited

Next steps

- Develop transfection protocols for other cell types
- Validate utility of parametric and non-parametric fitting
- Characterize engineered bone and cartilage
- Investigation of broader range of cellular responses
- Develop new ECM reference surfaces



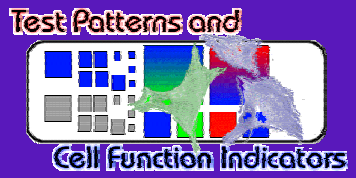
Building the Infrastructure

Polymers Division infrastructure

- Renovated 2000 ft^2 for biomaterial-library preparation, cell biology and imaging laboratories.
- Expand efforts through hiring staff and post-doctoral associates.

Biotechnology Division infrastructure

- Capitalize on existing thin film fabrication and analysis capabilities.
- Expand capabilities and expertise in molecular and cell biology by hiring Kurt Langenbach.



Strategic Partnerships

Establish strategic partnerships outside of NIST

Consultation on Gradient Libraries: ●NIH (Kenneth Yamada), ●UPenn (Paul Jamney), ●Integra Life Sciences (Fred Cahn), ● NIH (Rocky Tuan)

Consultation on Biomarkers: ●Osiris (Mark Pittenger), ●Echelon (Glen Prestwich), ●Advanced Tissue Sciences (J. Mansbridge), ● IME, UPenn (Peter Davies), ●UCHSC (Peter Jones)

Other collaborations

- Robert Levi, CHOP (chemically modified stents); ● Joachim Kohn, Rutgers (Preparation of new gradient libraries); ● Richard Spencer, NIH (Characterization of engineered cartilage); ● Clontech (Specific, high efficiency vectors); ● ReGen Corp (Collagen structure and function); ● Richard Gross, Brooklyn Polytechnic (High-throughput investigation of protein adsorption); ● Becton-Dickinson (Extracellular matrix substrates for comparison testing); ● Leica (Evaluation of serum protein interaction with materials); ASTM Workshop on Metrology for Cell Signaling ● Development of reference scaffolds with ASTM involvement

By working with strategic partners we will provide measurement tools that industry can use to qualify materials, cells, and manufacturing practices